Expression of Activated N-ras in a Primary Melanoma Cell Line Counteracts Growth Inhibition by Transforming Growth Factor-β

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One critical factor in melanoma progression is the change from radial growth phase to vertical growth phase. We previously showed a high incidence of *ras* mutations in progressing but not early human melanomas. We also found that stable expression of activated Ras in a primary human melanoma cell line (WM35) led to enhanced proliferation, anchorage-independent survival, migration and invasion *in vitro* and enhanced subcutaneous tumor formation *in vivo*, transforming the melanoma phenotype from the radial growth phase to the vertical growth phase. Inhibitory cytokines, especially transforming growth factor-β, are important in homeostasis of normal human melanocytes. Proliferation of early melanoma cells can be inhibited by transforming growth factor-β, whereas more aggressive stages lose this response. Using a transforming growth factor-β activated luciferase reporter transiently transfected into WM35, WM35N-ras, and WM35H-ras (WM35 transfected with mutant N-*ras* or H-*ras* genes), we demonstrated significant decreases ($p < 0.04$) in transforming growth factor-β induced reporter expression in both *ras* transfected cell lines. Transforming growth factor-β also induced significant decreases ($p < 0.002$) in the proportion of WM35 cells in S-phase of the cell cycle; this effect was not observed in WM35N-ras cells. Furthermore, we demonstrated that an important controlling factor in transforming growth factor-β inhibition of cell cycle progression, the phosphorylation of the Rb protein, was altered in WM35N-ras; transforming growth factor-β caused a marked relative increase in hypophosphorylated pRb in WM35 cells, but not in WM35N-ras. These data suggest that activated Ras plays an important part in melanoma progression from the radial growth phase to the vertical growth phase by counteracting inhibition by cytokines such as transforming growth factor-β, thus providing a growth advantage. *J Invest Dermatol 114:1200–1204, 2000*

Malignant melanoma is the most deadly skin cancer because it frequently metastasizes, often to the brain, is resistant to radio- and chemotherapy, and may reappear even 20 y after excision of the primary tumor. Melanoma provides a classic example of multistage carcinogenesis, showing well-defined stages of progression with distinct clinical, biologic, and histologic characteristics (Herlyn *et al*, 1987). Beginning as an *in situ* tumor arising in a common nevus, congenital nevus, or dysplastic nevus, primary melanoma progresses through a slowly growing radial growth phase (RGP), to a rapidly growing and invasive vertical growth phase (VGP). Melanomas from RGP or very early VGP have low metastatic potential, whereas those from late VGP have high risk for metastasis (Herlyn *et al*, 1985). This RGP-VGP functional switch is important in understanding invasive and metastatic potential of malignant melanoma.

Mutations in a *ras* allele occur in 30% of all human tumors, making *ras* the most widely mutated human proto-oncogene (Bos, 1989; Macara *et al*, 1996; McCormick and Wittinghofer, 1996). In our previous study of 100 melanomas, we found that none of the *in situ* melanomas had *ras* mutations, but 38% of invasive melanomas possessed *ras* mutations (Ball *et al*, 1994). Despite some reports of *ras* mutation in primary melanomas, associated with sun exposure and perhaps geographic factors (van Elsas *et al*, 1996; Jiveskog *et al*, 1998), other studies have concluded that most of the activating *ras* mutations occur during progression in melanoma, suggesting that ras mutations are not required for melanoma initiation (Albino *et al*, 1989; Jafari *et al*, 1995). We hypothesized that *ras* gene mutations may play an important part in the progression of RGP melanoma cells to VGP, invading the dermis.

To study the role of activated *ras* mutations in melanoma progression, we previously transfected mutant N-*ras* or mutant H-*ras* genes stably into WM35 cells, a human melanoma cell line of low invasive potential, derived from RGP melanoma. Expression of these *ras* mutants in WM35 cells produced cell lines with characteristics typical of the VGP of melanoma: anchorage-independent proliferation and survival, increased motility and invasiveness in *in vitro* assays, and subcutaneous tumor formation in immunodeficient mice (Fujita *et al*, 1999).

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Abbreviations: pRb, retinoblastoma gene product; RGP, radial growth phase; VGP, vertical growth phase; WM35H-ras and WM35N-ras, WM35 cells transfected with, respectively, mutant H-*ras* and mutant N-*ras* expression plasmids

Normal melanocyte proliferation is inhibited by a number of cytokines present in the epidermis, including interleukins 1 and 6, tumor necrosis factor-α, and TGF-β (Krasagakis *et al*, 1995). Kerbel has proposed that loss of this inhibition is a key progression factor in the switch of melanoma to the progressive phenotype (Kerbel *et al*, 1996). TGF-β is an important factor influencing cell proliferation, differentiation, and tumorigenesis (Polyak, 1996). This cytokine is a growth inhibitor for most cell types and inhibition is mediated, at least in part, by activation of cyclin-dependent kinase inhibitors including p15, p21, and p27 (Hannon and Beach, 1994; Reynisdottir *et al*, 1995; Florenes *et al*, 1996). Whereas cells from primary tumors often retain inhibition by TGF-β, this response is lost in more advanced malignancies, including metastatic melanoma (Florenes *et al*, 1996; Rodeck *et al*, 1994), as well as in various cells transfected with activated *ras* genes (Houck *et al*, 1989; Filmus *et al*, 1992) or microinjected with activated Ras protein (Howe *et al*, 1993). Our hypothesis is that *ras* mutations can contribute to progression by abrogating cytokine inhibition. WM35 cells retain a growth inhibitory response to TGF-β (Florenes *et al*, 1996). In this study, we report that the growth inhibitory effect of TGF-β is released by expression of mutant N-ras in this RGP melanoma cell line.

METHODS AND MATERIALS

Cell lines and culture conditions The cell line WM35 (Satyamoorthy *et al*, 1997), derived from a human primary, RGP melanoma was kindly provided by M. Herlyn (Wistar Institute). WM35N-ras and WM35H-ras were previously derived by stably transfecting activated N-*ras* (codon 12, Gly to Asp) or H-*ras* (codon 12, Gly to Val) into WM35 cells (Fujita *et al*, 1999). The cell lines were maintained as monolayers in RPMI1640 (GIBCO BRL, Baltimore, MD) with 10% NuSerum (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA). In a previous study (Fujita *et al*, 1999), we found no differences between the WM35 parent cell line and WM35 transfected with empty vector as a control in anchorage-dependent cell growth on tissue culture plastic, in cell movement, or in *in vitro* invasiveness. In this study, we therefore compared only the WM35 parent cell line with the N-*ras* or H-*ras* transfected cells.

TGF-β Transcriptional response assays Cells were transiently transfected with plasmid p3TP-Lux (Wrana *et al*, 1992; Attisano *et al*, 1993), using electroporation (Maxwell and Maxwell, 1988). Briefly, cells were detached with ethylenediamine tetraacetic acid (EDTA) and were suspended in complete medium at a concentration of 1×10^7 per ml. Samples (100 μl) were mixed with plasmid DNA (5 μg total, in 5 μl water) and were electroporated (IBI Gene Zapper, Eastman-Kodak) in a 4 mm cuvette (Bio-Rad, Hercules, CA) at 250 μfarad and 250 V, giving a time constant of about 30 ms. The electroporated cells were immediately split into two equal aliquots (2.5 ml) in a 6-well plate, with or without TGF-β1 (Genzyme Corporation, Cambridge, MA; final concentration 2.5 ng per ml) and incubated overnight. Luciferase activity was then measured using the Luciferase assay system (Promega, Madison, WI) in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

Cell cycle analysis by flow cytometry For cell cycle analysis, 5×10^4 cells were seeded in six-well plates with or without TGF-β1 (2.5 ng per ml) at time 0. The medium was replaced by fresh medium ± TGF-β after 18 h, 68 h, and 118 h. At the indicated time points, cells were harvested from individual wells by scraping into 0.5 ml Krishan's stain (1.12 mg per ml sodium citrate dihydrate, 0.0461 mg per ml propidium iodide, 0.01% NP40, 0.01 mg per ml RNase A). The samples were placed at 4°C overnight before cell cycle analysis using an Epi CS-XL flow cytometer (Beckman-Coulter Corp., Miami, FL) and ModFit LT Cell Cycle Analysis Software (Verity Software, Topsham, ME).

Immunoblot Cells (10^6) were harvested, washed with phosphate-buffered saline, suspended in lysis buffer (100 μl) (50 mM HEPES, 150 mM NaCl, 2.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, with protease inhibitors), and sonicated for 3× 10 s. The cell extracts were clarified by centrifugation at $10{,}000 \times g$ for 10 min and supernatants were subjected to polyacrylamide gel electrophoresis (Laemmli system), loading equal amounts (40 μg) of total protein. Proteins were transferred to PVDF membrane (Millipore, Bedford, MA) using a Semi-Dry transfer system (Bio-Rad, Hercules, CA), and probed with specific antibodies against pRb (Pharmingen, San Diego, CA) at 1:500 dilution. Chemiluminescence detection used peroxidase-conjugated secondary antibodies and an ECL kit (Amersham, Arlington Heights, IL). Exposed films were scanned, and bands were quantitated with software Quantity One (Bio-Rad).

RESULTS

Activated Ras abrogates transcriptional response to TGF-β To study the effect of activated Ras on TGF-β signaling activity, we assayed the ability of TGF-β to induce activation of the p3TP-Lux reporter construct (Wrana *et al*, 1992; Attisano *et al*, 1993), transiently transfected into WM35, WM35H-ras, or WM35N-ras cells. Plasmid p3TP-Lux contains a region from the

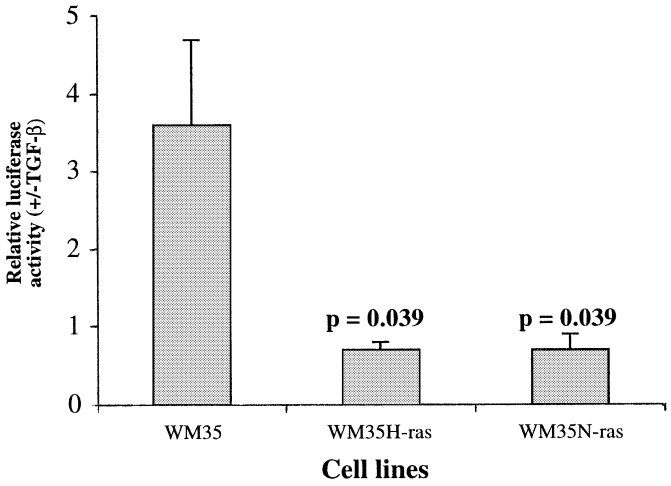

Figure 1. Activated Ras abrogates transcriptional response to TGF-β in WM35 cells. Assays for transcriptional response were performed using WM35, WM35H-ras, and WM35N-ras cells, transiently transfected with p3TP-lux, with or without TGF-β treatment as described in *Materials and Methods*. Data were plotted as fold increase in luciferase activity in cells treated with TGF-β *vs* cells without TGF-β treatment. p-values shown between WM35 and WM35N-ras, or between WM35 and WM35H-ras were from the Student's t test. The activities measured without TGF-β were (light units per 10^6 cells): 11.96 ± 2.25, 23.16 ± 9.58, 6.46 ± 1.29 for WM35, H-ras, and N-ras, respectively,

Table I. Activated Ras abrogates growth inhibitory effect of TGF-β on WM35 cells[a]

	WM35			WM35N-ras		
	(%)G1	(%)G2/M	(%)S	(%)G1	(%)G2/M	(%)S
–TGF-β	65.3	18.5	14.5	71.9	13.8	14.3
+TGF-β	70.6	19.8	9.6	72.2	12.6	15.2
t-test (+/–)	0.0119[b]		0.0017[b]			

[a]This table shows the percentage of cells in different cell cycle stages, averaged over the 11 time points.
[b]Only these are significantly different.

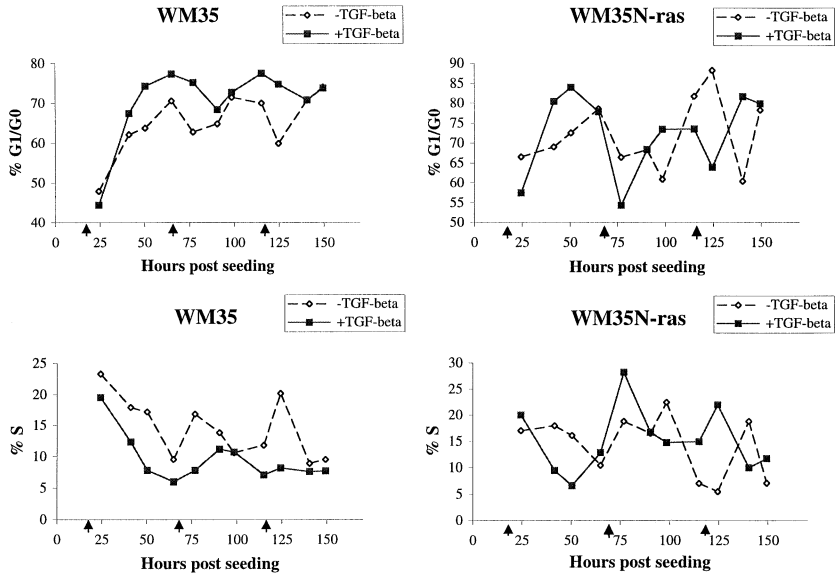

Figure 2. Activated Ras abrogates growth inhibitory effect of TGF-β on WM35 cells. WM35 and WM35N-ras cells, cultured with or without TGF-β, were harvested at the indicated time points and subjected to cell cycle analysis by flow cytometry. Arrows mark medium changes.

human plasminogen activator inhibitor 1 gene promoter and three tetradecanoyl phorbol acetate-responsive elements. Luciferase reporter gene expression from this promoter was previously shown responsive to TGF-β in transiently transfected cells (Attisano *et al*, 1993; Wrana *et al*, 1992). As shown in **Fig 1**, TGF-β-induced luciferase activity in WM35 cells more than 3-fold, whereas there was no induction in WM35N-ras or WM35H-ras cells, suggesting that activated Ras abrogates TGF-β transcriptional control of gene expression.

Activated Ras alleviates growth inhibitory effect of TGF-β on WM35 Previous studies (Rodeck *et al*, 1994) have shown that early stage human melanoma cell lines, including WM35, are growth inhibited by TGF-β. We have confirmed this observation by cell cycle analysis using flow cytometry. We treated WM35 and WM35N-ras cells with 2.5 ng per ml TGF-β for up to 150 h, harvesting samples at various time points for cell cycle analysis. The results are shown in **Fig 2** and summarized in **Table I**. **Table I** shows the percentage of cells in different cell cycle stages, averaged over the 11 time points. There are significant differences in percentage of cells in G_1/G_0 ($p = 0.012$) or S-phase ($p = 0.0017$) in WM35 cells with, *vs* without, TGF-β treatment (**Table I**). Treatment of WM35 cells with TGF-β led to a substantial overall decrease of the proportion of cells in S-phase, with a concomitant accumulation in G_0/G_1 phase (**Table I, Fig 2**). In contrast, WM35N-ras cells showed no comparable changes in cell cycle distribution and, especially, no decrease of cells in S-phase during TGF-β treatment (**Table I, Fig 2**). Although no deliberate attempt was made to synchronize the cells used in these experiments, it is apparent from the periodic fluctuation of the percentage of cells in S-phase that a substantial degree of synchronization occurred for both WM35 and WM35N-ras (**Fig 2**). We have consistently observed this effect with these melanoma cell lines following plating of cells detached using trypsin plus EDTA, or even EDTA alone. The apparent synchronization may have been enhanced in the experiment presented in **Fig 2** by a proliferation response to the medium changes at the times indicated by arrows. For WM35 cells, this response was clearly blunted when TGF-β was included in the medium. It is interesting to note that an opposite effect occurred in the WM35N-ras cells, in which S-phase entry apparently occurred sooner following changes of medium containing TGF-β. These results suggest that the WM35N-ras cells had not only lost the negative proliferation response to TGF-β but may also, to some extent, have acquired a positive response to this cytokine manifest by earlier S-phase entry after medium change.

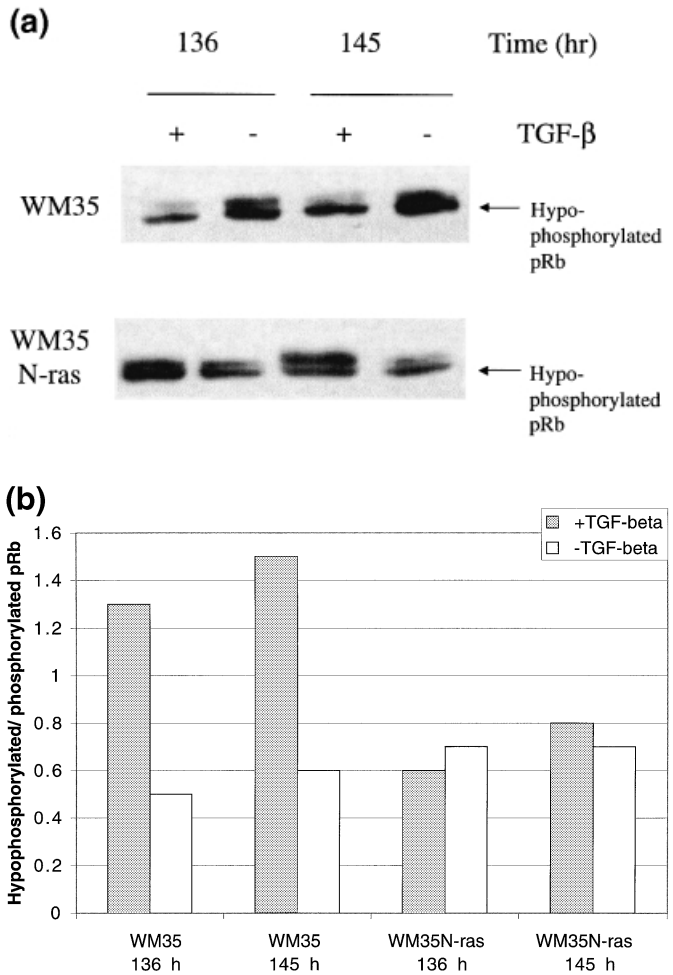

Figure 3. Relative increase in hypophosphorylated pRb in WM35, but not in WM35 N-ras cells treated with TGF-β. (*A*) pRb immunoblot of samples harvested at the indicated times post seeding (corresponding with late time points in **Fig 2**). (*B*) Quantitation of *A* results. Y-axis shows the ratio of hypophosphorylated-pRb/hyperphosphorylated-pRb in each lane of *A*.

Activated Ras inhibits induction of hypophosphorylated pRb by TGF-β in WM35 cells Phosphorylation of the pRb protein is a requirement for cell cycle progression through G_1 into S-phase (Buchkovich *et al*, 1989; Chen *et al*, 1989; DeCaprio *et al*, 1989; Cobrinik *et al*, 1992; Hinds *et al*, 1992). We investigated the phosphorylation status of pRb in WM35 and WM35N-ras cells with or without TGF-β treatment. The immunoblot is shown in **Fig 3**(***A***). We quantitated the hypo- and hyperphosphorylated pRb bands, and plotted the data as the ratio of hypo/hyperphosphorylated pRb in each lane (**Fig 3*B***). Treatment of WM35 melanoma cells with TGF-β increased this ratio by more than 2-fold, whereas the same treatment of WM35N-ras cells resulted in no relative increase in hypophosphorylated pRb (**Fig 3*B***).

DISCUSSION

The loss of response to negative growth factors is one stage in tumor progression (Sporn and Roberts, 1985). Loss of growth inhibition by TGF-β has frequently been observed in cell lines established from many human tumors, including adenocarcinomas, gliomas, and late-stage melanomas (Fynan and Reiss, 1993). Cells from early stage melanomas, however, can be inhibited by TGF-β (Rodeck *et al*, 1994). The results presented here indicate that introduction of activated Ras into a primary melanoma cell line confers resistance to the inhibitory effects of TGF-β. Similar observations have been made in rat liver epithelial cells, parenchymal hepatocytes, and rat intestinal cells (Houck *et al*, 1989; Filmus *et al*, 1992). Thus, an important part of the role of activated Ras in tumorigenesis may lie in abolishing response to negative growth factors. Both the retinoblastoma gene product (pRb) and cyclin-dependent kinase inhibitors have been implicated in the response to TGF-β signaling (Alexandrow and Moses, 1995). Hypophosphorylated pRb prevents progression through the cell cycle by sequestering E2F transcription factors. TGF-β appears to prevent phosphorylation of pRb by increasing expression of cyclin-dependent kinase inhibitors, including $p15^{INK4B}$, $p21^{waf1/cip1}$, and $p27^{kip1}$. Induction of the latter, kip family cyclin-dependent kinase inhibitors is apparently sufficient for G_1 growth arrest in WM35 cells, in which $p15^{INK4B}$ is inactive (Florenes *et al*, 1996). Our present results suggest that activated Ras abrogates the growth inhibitory effects of TGF-β by directly or indirectly preventing the accumulation of hypophosphorylated pRb.

Although we have not directly addressed the mechanism of the loss of a TGF-β negative response, it seems unlikely that this was due to loss of functional cell surface receptors. Using specific antibodies, we were unable to detect any consistent differences between WM35 and WM35N-ras in expression levels of any of the three components (receptors types I, II, or III; Graulich *et al*, 1999) of the TGF-β receptor complex (unpublished data). Maintenance of functional receptor would also be consistent with our data (**Fig 2**) suggesting that WM35N-ras cells retain some response to TGF-β, which is not reflected by growth inhibition. The pathway of signal transduction in response to TGF-β involves phosphorylation of the proteins smad 2 and smad 3 by the kinase activity of the type I receptor, followed by their association with smad 4 in a complex, which is translocated to the nucleus and then serves to activate specific gene transcription (Wrana and Pawson, 1997). It was recently reported that expression of activated Ras in epithelial cells resulted in phosphorylation of smads 2 and 3 at additional sites that prevented their nuclear translocation, thus inhibiting smad-mediated signal transduction (Kretzschmar *et al*, 1999). Evidence, however, was also recently presented for the maintenance of smad-mediated transcription in several advanced or metastatic melanoma cell lines that had lost growth inhibition response to TGF-β (Rodeck *et al*, 1999). Our observation of the failure of TGF-β to induce reporter expression from p3TP-lux in WM35N-ras and WM35H-ras cells (**Fig 1**) would be consistent with the inactivation of smad signaling by activated Ras in WM35 cells; however, further work is needed to substantiate this inference.

Previously, we found that expression of activated *ras* mutants in WM35 cells produced characteristics typical of the VGP of melanoma: anchorage-independent proliferation and survival, increased motility and invasiveness in *in vitro* assays, and subcutaneous tumor formation in immunodeficient mice (Fujita *et al*, 1999). Our current data suggest that activated Ras may also provide a growth advantage *in vivo* in part by abrogating the response to negative growth factors such as TGF-β. In other studies, we found that activated Ras could provide a survival advantage to the same cell lines by increasing resistance to apoptosis (manuscript submitted). Therefore, activated Ras enhances melanoma progression by providing several mechanisms involving both growth and survival advantages.

Recent studies have demonstrated an important role for activated Ras in melanoma genesis and maintenance in a murine model (Chin *et al*, 1997; Chin *et al*, 1999). First, the targeted expression of activated H-Ras in the melanocytes of mice null for $p16^{INK4A}$ resulted in a high frequency of melanomas (Chin *et al*, 1997). Second, continued expression of activated Ras was necessary for maintenance of these melanomas, as demonstrated using a conditional (doxycycline-induced) expression system. Thus, H-*ras* downregulation upon withdrawal of doxycycline resulted in clinical and histologic regression of primary and explanted tumors (Chin *et al*, 1999). Our data complement these studies by highlighting the important role of activated Ras in the development of human melanoma. These results suggest that Ras antagonists could prove valuable for melanoma therapy.

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